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Research article

Suitability of the microbial community composition and function in a semiarid mine soil for assessing phytomanagement practices based on mycorrhizal inoculation and amendment addition

J. Kohler [a, b], F. Caravaca [c, *], R. Azcón [d], G. Díaz [e], A. Roldán [c]

[a] Freie Universität Berlin, Institut für Biologie, Plant Ecology, Altensteinstr. 6, D-14195, Berlin, Germany
[b] Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), D-14195, Berlin, Germany
[c] CSIC-Centro de Edafología y Biología Aplicada del Segura, Department of Soil and Water Conservation, P.O. Box 164, Campus de Espinardo, 30100, Murcia, Spain
[d] CSIC-Estación Experimental del Zaidín, Microbiology Department, Profesor Albareda, 1, 18008, Granada, Spain
[e] Universidad Miguel Hernández de Elche, Department of Applied Biology, Avda. Ferrocarril, s/n. Edf. Laboratorios, 03202, Elche, Alicante, Spain

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ABSTRACT

The recovery of species composition and functions of soil microbial community of degraded lands is crucial in order to guarantee the long-term self-sustainability of the ecosystems. A field experiment was carried out to test the influence of combining fermented sugar beet residue (SBR) addition and inoculation with the arbuscular mycorrhizal (AM) fungus *Funneliformis mosseae* on the plant growth parameters and microbial community composition and function in the rhizosphere of two autochthonous plant species (*Dorycnium pentaphyllum* L. and *Asteriscus maritimus* L.) growing in a semiarid soil contaminated by heavy metals. We analysed the phospholipid fatty acids (PLFAs), neutral lipids fatty acids (NLFAs) and enzyme activities to study the soil microbial community composition and function, respectively. The combined treatment was not effective for increasing plant growth. The SBR promoted the growth of both plant species, whilst the AM fungus was effective only for *D. pentaphyllum*. The effect of the treatments on plant growth was linked to shifts in the rhizosphere microbial community composition and function. The highest increase in dehydrogenase and β-glucosidase activities was recorded in SBR-amended soil. The SBR increased the abundance of marker PLFAs for saprophytic fungi, Gram+ and Gram− bacteria and actinobacteria, whereas the AM fungus enhanced the abundance of AM fungi-related NLFA and marker PLFAs for Gram− bacteria. Measurement of the soil microbial community composition and function was useful to assess the success of phytomanagement technologies in a semiarid, contaminated soil.
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1. Introduction

Phytostabilization of abandoned mine lands based on the use of native metal-tolerant plant species with different functional traits, such as shrubs and grasses, has been recommended for the establishment of a self-sustaining plant community, allowing the further recovery of such degraded sites (Parraga-Aguado et al., 2014). Among the technologies for implanting a permanent vegetation cover in semiarid mine tailings, inoculation with plant-growth-promoting microorganisms and the application of newly-developed organic amendments to the soil can be a suitable approach (Gamalero et al., 2009; Fernández et al., 2012). Arbuscular mycorrhizal fungi (AMF) may protect their host plants from the toxicity of excessive metal concentrations through direct hyphal sequestration and accumulation of metals or by indirectly improving P nutrition under such harmful conditions (Giasson et al., 2008; Meier et al., 2012). The AMF are able to colonise heavy-metal-contaminated soils, although their diversity and abundance usually decrease with increasing heavy metal content, and some strains are more heavy-metal-resistant than others (Zarei et al., 2010).

The establishment of plants on mine tailings generally requires the input of an organic residue, to alleviate the toxicity of the tailings and improve soil fertility (Mendez and Maier, 2008). Organic amendments are known to increase the metal complexation and adsorption, decreasing the availability of heavy metals to

* Corresponding author.
E-mail address: fcb@cebas.csic.es (F. Caravaca).

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plants (de la Fuente et al., 2011). In addition, organic residues such as *Aspergillus niger*-treated sugar beet agrowaste (SBR) are able to improve the structural stability of mine tailings in semiarid ecosystems as well as plant growth (Carrasco et al., 2009; Kohler et al., 2014). The efficacy of organic amendments has been proven in both mesocosms (Pérez-de-Mora et al., 2006) and field assays (Pérez-de-Mora et al., 2011; Pardo et al., 2014). The combination of AM fungal inoculation and organic waste amendment as a phytomanagement technology in heavy-metal-polluted soils has been researched only scarcely, but there are some indications that demonstrate its effectiveness with regard to maximising the success of revegetation (Wang et al., 2013). However, the efficacy of combining these two technologies has not been demonstrated under field conditions.

Excessive levels of heavy metals may constitute a serious hazard for soil microorganisms – affecting their growth, activity and composition (Giller et al., 2009; Mandal et al., 2014). Considering that soil microbial populations are essential to nutrient cycling and plant nutrient availability, the recovery of the composition and activity of microbial communities may be key to the sustainability of mine ecosystems. Meanwhile, the composition and activity of soil microbial populations have been proposed as useful indicators of the improvement of soils contaminated by heavy metals following the implementation of phytomanagement technologies (Pérez-de-Mora et al., 2006). We have previously shown, in a greenhouse pot assay, that the populations of AMF change in response to the addition of an organic amendment to a contaminated soil (Alguacil et al., 2011). Changes in the bacterial community composition have been reported also in mine soils subjected to different amendments (Pérez-de-Mora et al., 2006; Zornoza et al., 2015), which may also be modulated by the soil moisture regime (Fernández et al., 2012). However, there are relatively few studies that demonstrate the usefulness of measuring such microbiological properties during phytomanagement tasks under semiarid conditions.

Therefore, we hypothesised that the fermented SBR and/or the inoculation with a native AM fungus may alter the microbial community composition, resulting in the establishment of a fully-functional population that could favour plant growth. The objective of this study was to evaluate in a field experiment the influence of combining the addition of fermented SBR and the inoculation with AM fungus *Funneliformis mosseae* on the plant growth parameters and on composition and functions of microbial communities in the rhizosphere of two Mediterranean plant species (*Dorycnium pentaphyllum* and *Asteriscus maritimus*) growing in a semiarid soil contaminated by heavy metals. The soil microbial community composition and functions were assessed by measuring phospholipid fatty acids (PLFAs) and AM fungi-related neutral lipid fatty acid (NLFA) and enzyme activities involved in the cycling of carbon and phosphorus, respectively. The information obtained will permit determining whether such microbiological properties, related to the functioning and maintenance of ecosystems, can be used as indicators to monitor and judge the suitability of phytomanagement practices.

2. Materials and methods

2.1. Study site

This research was conducted on a mine tailing mound (37°35′33.2″ N, 0°52′35.5″ W, length: 200–300 m, width: 95 m, height: 25 m, volume: 750,000 m^3) at The Cartagena–La Unión mining district "Sierra Minera" (SE Spain). The ore deposits of the mine tailings contain Fe, Pb and Zn as main heavy metal components. The climate is semiarid Mediterranean with a mean annual precipitation of 275 mm, a mean annual temperature of 17.5 °C and a mean potential evapo-transpiration of 1000 mm. For soil characterization, we randomly took three soil samples from 0 to 20 cm depth each consisting of a mixture of six subsamples. Initial characteristics of mine tailing soil are shown in Table 1.

2.2. Materials

The plants used were *D. pentaphyllum* Scop. and *A. maritimus* (L.) Less. The woody legume *D. pentaphyllum* is highly mycorrhizal and responded well to organic amendment (Caravaca et al., 2004). Its use for phytostabilization of heavy metal-contaminated areas was proposed by Lefèvre et al. (2009) due to its resistance to Cd and Zn. *A. maritimus* (synonym *Pallenis maritima*) is an herbaceous perennial halophyte with a high dependence of AM fungi (Estrada et al., 2013). It is a representative plant species in arid and saline Mediterranean ecosystems, found generally in rocks and stony slopes. Prior to the experimental procedures, *D. pentaphyllum* and *A. maritimus* seedlings were grown for 1 year in nursery conditions with peat as substrate. At planting, *D. pentaphyllum* and *A. maritimus* were 51.2 ± 12.2 and 17.5 ± 1.3 cm high, respectively with a shoot dry mass of 1.50 ± 0.29 and 1.69 ± 0.36 g, respectively ($n = 5$).

The mycorrhizal inoculum was a *F. mosseae* (former *Glomus mosseae*) strain, being the most abundant AMF in the mine tailing (Azcón et al., 2009). The mycorrhizal inoculum was multiplied using trap cultures of *Sorghum bicolor* (L.) Moench, and consisted of rhizospheric soil, spores, hyphae and infected root fragments.

Sugar beet residue (SBR) was inoculated with *A. niger* strain NB2 and rock phosphate (Morocco fluorapatite, 12.8% P, 1 mm mesh) by solid state fermentation (Kohler et al., 2014). The concentration of P was determined after nitric-perchloric acid digestion using an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo electron corporation Mod. IRIS intrepid II XDL). Total N was determined by dry combustion using a LECO Tru-Spec CN analyzer (Leco Corp., St. Joseph, MI, USA). The main characteristics of fermented SBR are described in Kohler et al. (2014).

2.3. Experimental design and layout

The experiment was conducted as a complete randomised factorial design with two factors. The first factor had two levels: non-addition or addition of fermented SBR; and the second had two levels: non-inoculation or inoculation with *F. mosseae*. In the experimental area, planting holes 10 × 10 cm wide and 20 cm deep

Table 1
Physico-chemical and chemical characteristics of the soil used in the experiment (N = 3).

pH (H_2O)	7.7 ± 0.3
EC (1:5, dS m^{-1})	2.5 ± 0.4
$CaCO_3$ (%)	<5
Total organic C (g kg^{-1})	4.3 ± 0.6
Total N (g kg^{-1})	0.21 ± 0.03
Clay (%)	5 ± 2
Silt (%)	24 ± 5
Sand (%)	71 ± 6
Aggregate stability (%)	24.7 ± 1.6
Fe_2O_3 (%)	16 ± 1
Al_2O_3 (%)	8 ± 1
Total Zn (mg kg^{-1})	12100 ± 900
Soluble Zn (mg kg^{-1})	192 ± 62
Total Pb (mg kg^{-1})	8950 ± 300
Soluble Pb (mg kg^{-1})	3 ± 1
Total Cu (mg kg^{-1})	221 ± 20
Soluble Cu (mg kg^{-1})	<0.01
Total Cd (mg kg^{-1})	61 ± 11
Soluble Cd (mg kg^{-1})	1 ± 0

were dug manually and treatments were randomly assigned to them. SBR was added to soil at a rate of 1.2% (w/w), whereas the mycorrhizal inoculum was applied at a rate of 5% (v/v). For this, the mycorrhizal inoculum and organic residue were manually mixed with 2 kg of soil in plastic bags and introduced in the plantation holes. The same amount of the autoclaved inoculum was also mixed with the soil of non-inoculated plants. The microbial populations accompanying the mycorrhizal fungi were added to non-inoculated plants using a filtrate (Whatman no. 1 paper) of mycorrhizal inoculum. In October 2012, *D. pentaphyllum* and *A. maritimus* seedlings were planted in individual holes with at least 1 m between holes and with 3 m between treatment levels. At least 25 seedlings per treatment level were planted for each plant species.

2.4. Sampling procedures

Eight months after planting, five plants per treatment including root systems and soil firmly adhering to roots (rhizosphere soil) were harvested. A total number of 20 plants were collected for each plant species. For separating the rhizosphere soil from the root system, samples were shaken into a plastic bag. Rhizosphere soil samples were divided in two subsamples: one subsample was dried at room temperature and sieved at 2 mm for physical-chemical and chemical analyses and the other subsample sieved at 2 mm and stored for up to 2 weeks at 4 °C for biochemical analyses. An aliquot of field-moist soil samples was frozen at −20 °C and stored until PLFA and NLFA analyses.

2.5. Plant analyses

Dry (105 °C, 5 h) mass of shoots and roots biomass were measured. Shoot tissues were finely ground before chemical analysis. Shoot P, K, Cd, Cu, Pb and Zn were quantified in the HNO_3—$HClO_4$ digestion extract using an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo electron corporation Mod. IRIS intrepid II XDL). The precision and accuracy of this method were tested using the CTA-VTL-2 certified material (Virginia Tobacco leaves). Heavy metal recoveries from plant standards ranged between 89 and 110% for the elements analysed. N in shoot tissues was determined by dry combustion using a CN analyzer (Leco Corp., St. Joseph, MI, USA).

The mycorrhizal colonization of roots was determined by the gridline intersect method (Giovanetti and Mosse, 1980) after clearing with KOH and staining with trypan blue (Phillips and Hayman, 1970).

2.6. Soil analyses

Total N was measured by the Kjeldahl method. Available P was measured in a sodium bicarbonate extract (1:20 w/v) using a spectrophotometer (Watanabe and Olsen, 1965). Total metal concentrations were quantified using an ICP-MS (Thermo electron corporation Mod. IRIS intrepid II XDL) after wet acid digestion of soil samples (Kohler et al., 2014). Soluble metal were measured in an aqueous extract (1:5 w/v) (Ernst, 1996). The accuracy of this methodology was assessed using a CRM027-050 Certified Material (Resource Technology Corporation, USA). The recoveries from soil standard were between 84 and 112% for the elements analysed.

Dehydrogenase activity was determined using INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) as substrate following the method of García et al. (1997).

Acid phosphomonoesterase activity was determined using *p*-nitrophenyl phosphate disodium as substrate according to Naseby and Lynch (1997).

β-glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG, 0.05 M) as substrate following the procedure described by Tabatabai (1994).

PLFA extraction and analysis was performed following the procedure described in Frostegård and Bååth (1996). Briefly, lipids were extracted from a fresh soil sample equivalent to a dry matter of 5 g with a chloroform-methanol-citrate buffer (1:2:0.8). After extraction the lipids were fractionated into neutral lipids (NLFA), glycolipids and polar lipids (PLFAs) on a silica column. Neutral lipids and phospholipids were methylated, and the fatty acid methyl esters were separated by gas chromatography equipped with a flame ionization detector. Peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard. Separated fatty acid methyl-esters were identified by chromatographic retention time and mass spectral comparison using standards qualitative bacterial acid methyl ester mix #47080-U and 37-component FAME mix #47885-U supplied by Supelco (Bellefonte, USA) and methyl 10-methylhexadecanoate and methyl 10-methyloctadecanoate supplied by Larodan Lipids (Malmö, Sweden).

The soil microbial community composition was determined by using the following PLFAs: 18:2 ω6,9 and 18:1 ω9 for saprotrophic fungi, and i15:0, a15:0, i16:0, i17:0, a17:0, 17:0 10-meth, 18:0 10-meth, cy17:0, cy19:0, 16:1 ω7, 17:1 ω8 and 18:1 ω7 for bacteria (Frostegård and Bååth, 1996; Zelles, 1999). The NLFA 16:1 ω5 was assigned as marker for AMF (Olsson et al., 2003).

2.7. Statistical analysis

Percentage colonization was arcsin-transformed, and the other parameters were log-transformed to compensate for heterogeneity of variance, before analysis of variance, when necessary (shoot heavy metals). Mycorrhizal inoculation, organic amendment and their interactions effects on measured variables were tested by a two-way ANOVA and comparisons among means were made using the Tukey's HSD-test calculated at $p < 0.05$. For each plant species, the abundances of individual fatty acids were analysed by principal component analysis (PCA) in order to determine the changes in soil microbial community composition in response to the used phytoremediation technologies. In addition, analysis of variance (ANOVA) of the loading values of the individual PLFAs for PC1 and PC2, with the Tukey's HSD-test as post-hoc test, were used to test for significant differences in microbial community composition with phytomanagement. Correlation analysis between soil parameters measured and the scores for the first principal component of the PLFA data from both plants was carried out using Pearson's correlation coefficients. Statistical procedures were performed with the software package SPSS 12.0 for Windows.

3. Results

3.1. Effects of the amendment and AM fungus on plant growth, nutrients uptake and mycorrhizal colonisation

Both the addition of SBR and mycorrhizal inoculation significantly improved the shoot and root biomass of *D. pentaphyllum* (Fig. 1, $P = 0.044$ and $P = 0.004$ for shoot dry mass, respectively and $P = 0.040$ and $P = 0.028$ for root dry mass, respectively). The results of the ANOVA indicate that there was a significant amendment-mycorrhizal inoculation interaction for both growth parameters (AxM interaction, $P < 0.001$ and $P = 0.002$, respectively); the combined treatment significantly decreased root biomass compared to *F. mosseae*-inoculated plants and plants grown in the amended soil. The addition of SBR and AM fungus significantly increased the N and P contents of shoots with respect to the control

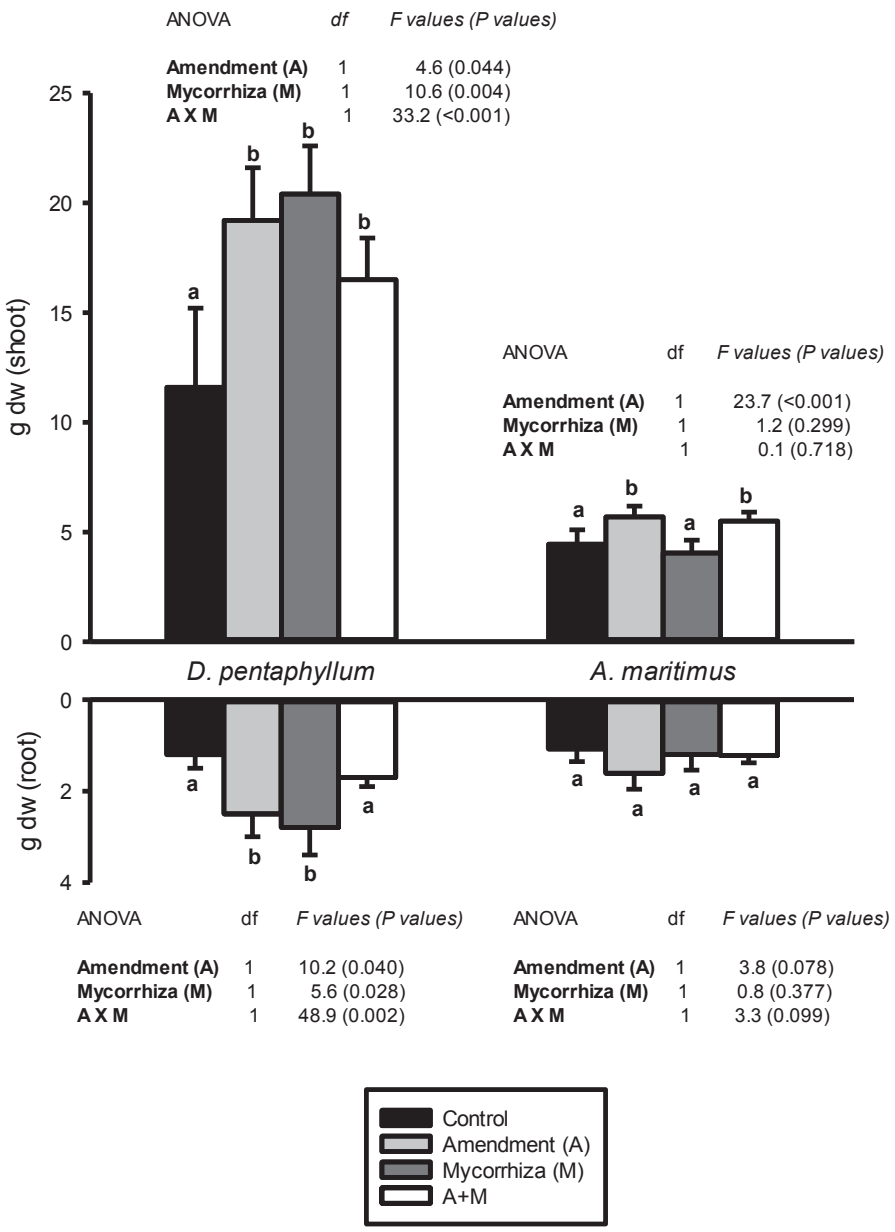

Fig. 1. Effects of fermented sugar beet residue addition and mycorrhizal inoculation on shoot and root dry mass of *D. pentaphyllum* and *A. maritimus*, eight months after planting (n = 5). For each plant species and each growth parameter, bars followed by the same letter are not significantly different according to the Tukey's HSD-test (P < 0.05). Bars represent standard deviations.

plants (Table 2), whilst only mycorrhizal inoculation enhanced the levels of shoot K (by 116% compared to control plants).

For *A. maritimus* plants, only the addition of SBR significantly increased shoot dry mass (by 28% compared to control plants). Neither the addition of SBR nor the inoculation with the AM fungus had a significant effect on root dry mass (Fig. 1). The shoot P was not affected significantly by any factor, whereas the shoot N and K contents were increased by the amendment (Tables 2 and 3).

In *A. maritimus*, root colonisation by AMF was generally lower than in *D. pentaphyllum* (Table 2). The inoculation with *F. mosseae* was effective at increasing the percentage of mycorrhizal colonisation in the roots of both plant species ($P < 0.001$), particularly in *A. maritimus* (Table 3).

3.2. Effects of the amendment and AM fungus on soil enzyme activities, nutrients and soluble metals

The dehydrogenase and β-glucosidase activities in the rhizospheric soil from *D. pentaphyllum* were enhanced by the amendment (Table 4, by 84% and 50%, respectively). However, neither the amendment nor the AM fungus had a significant effect on the phosphomonoesterase activity (Table 3). The ANOVA results revealed significant increases in the β-glucosidase ($P = 0.002$) and dehydrogenase ($P < 0.001$) activities in the rhizosphere of *A. maritimus* plants with the addition of the SBR, but there was no effect on the acid phosphomonoesterase activity (Table 3).

For both plant species, only the amendment caused significant increases in the concentrations of available P and total N. With the

Table 2
Macronutrients in shoots and mycorrhizal colonization in response to addition of fermented sugar beet residue (A) and AMF inoculation (M) of *D. pentaphyllum* and *A. maritimus* seedlings 8 months after planting (mean ± standard deviation, n = 5).

Parameters	*D. pentaphyllum*	*A. maritimus*
Shoot P (mg plant^{-1})		
C	6 ± 2	8 ± 3
A	11 ± 1	11 ± 4
M	10 ± 2	8 ± 2
A × M	13 ± 3	11 ± 3
Shoot N (mg plant^{-1})		
C	148 ± 52	82 ± 11
A	278 ± 51	127 ± 8
M	360 ± 44	76 ± 13
A × M	304 ± 33	133 ± 10
Shoot K (mg plant^{-1})		
C	80 ± 30	50 ± 6
A	136 ± 20	66 ± 12
M	173 ± 40	49 ± 7
A × M	151 ± 37	75 ± 12
Colonization (%)		
C	68 ± 6	11 ± 5
A	76 ± 3	18 ± 8
M	88 ± 4	38 ± 7
A × M	86 ± 4	44 ± 4

exception of Zn, the soluble concentrations of metals (Pb, Cd and Cu) were below the detection limit in the rhizosphere of both species (data not shown). However, neither the amendment nor the AM fungus had a significant effect on the concentration of soluble Zn in soil.

3.3. Effects of the amendment and AM fungus on the accumulation of heavy metals in shoots

The shoot concentrations of Pb and Zn of *D. pentaphyllum* plants were significantly ($P = 0.019$ and $P = 0.013$, respectively) increased by the inoculation with *F. mosseae* (on average, about 61% and 21%, respectively, with respect to control plants), as shown in Table 5. There was a significant interaction between the SBR and the AM fungus regarding the concentration of shoot Cu (Table 3, $P = 0.007$). Thus, the highest levels of Cu were recorded in the shoots of inoculated plants grown in the amended soil (Table 5).

In the shoots of *A. maritimus* the concentrations of Zn were the only ones significantly ($P = 0.050$) affected by the addition of SBR, being higher in plants grown in amended soil than in those from non-amended soil (Tables 3 and 5).

Table 3
Two factor ANOVA (fermented sugar beet residue addition (A) and AMF inoculation (M)) for plant nutrients, soil enzymatic activities, heavy metals and abundance of phospholipid fatty acids and AMF neutral lipid fatty acid biomarkers (F values (P values)).

	Amendment (A)	Mycorrhiza (M)	Interaction (A × M)
D. pentaphyllum			
Shoot P	18.4 (<0.001)	10.0 (0.005)	2.0 (0.175)
Shoot N	12.2 (0.007)	19.2 (<0.001)	1.5 (0.483)
Shoot K	2.8 (0.112)	20.0 (<0.001)	2.2 (0.178)
Colonization	0.2 (0.630)	18.6 (<0.001)	2.0 (0.167)
Dehydrogenase	155.4 (<0.001)	38.7 (<0.001)	0.7 (0.405)
Phosphomonoesterase	0.1 (0.729)	2.9 (0.106)	0.1 (0.979)
β-glucosidase	5.1 (0.035)	8.2 (0.010)	0.6 (0.436)
Available P	9.4 (0.006)	4.1 (0.066)	0.9 (0.359)
Total N	37.6 (<0.001)	0.7 (0.787)	0.1 (0.830)
Shoot Cd	0.7 (0.400)	1.1 (0.301)	0.8 (0.395)
Shoot Cu	0.8 (0.387)	0.7 (0.477)	38.9 (0.007)
Shoot Pb	3.6 (0.071)	6.5 (0.019)	0.2 (0.633)
Shoot Zn	1.9 (0.178)	7.4 (0.013)	1.0 (0.335)
Gram+ PLFA	30.6 (<0.001)	0.4 (0.509)	2.2 (0.152)
Gram− PLFA	29.9 (<0.001)	21.6 (<0.001)	0.7 (0.418)
Saprophytic fungi PLFA	6.5 (0.019)	0.4 (0.511)	0.3 (0.560)
AB PLFA	14.1 (<0.001)	1.4 (0.245)	0.1 (0.894)
AMF NFLA	0.5 (0.490)	21.7 (<0.001)	0.5 (0.483)
Total PLFA	27.3 (0.003)	0.3 (0.830)	1.1 (0.202)
A. maritimus			
Shoot P	3.9 (0.075)	0.1 (0.874)	0.0 (0.945)
Shoot N	24.3 (<0.001)	0.3 (0.530)	0.1(0.727)
Shoot K	14.9 (0.003)	0.7 (0.418)	0.9 (0.350)
Colonization	1.1 (0.345)	18.9 (<0.001)	2.1 (0.178)
Dehydrogenase	40.6 (<0.001)	0.9 (0.371)	0.1 (0.780)
Phosphomonoesterase	0.7 (0.409)	3.8 (0.077)	0.8 (0.397)
β-glucosidase	16.0 (0.002)	0.9 (0.362)	0.8 (0.382)
Available P	9.9 (0.009)	0.6 (0.464)	2.6 (0.135)
Total N	42.1 (<0.001)	0.6 (0.435)	0.5 (0.544)
Shoot Cd	0.6 (0.423)	0.6 (0.455)	0.4 (0.512)
Shoot Cu	1.9 (0.200)	0.1 (0.758)	0.2 (0.641)
Shoot Pb	3.7 (0.082)	1.6 (0.226)	5.2 (0.043)
Shoot Zn	4.7 (0.050)	1.3 (0.269)	3.7 (0.079)
Gram+ PLFA	38.9 (<0.001)	0.0 (0.859)	1.2 (0.289)
Gram− PLFA	21.6 (0.001)	0.9 (0.359)	0.1 (0.746)
Saprophytic fungi PLFA	15.3 (0.002)	0.1 (0.767)	0.5 (0.477)
AB PLFA	125.3 (<0.001)	28.0 (<0.001)	8.3 (0.015)
AMF NFLA	2.9 (0.118)	19.1 (<0.001)	3.7 (0.081)
Total PLFA	32.0 (0.001)	1.6 (0.179)	1.8 (0.137)

Table 4
Soil enzymatic activities, available P and total N in response to addition of fermented sugar beet residue (A) and AMF inoculation (M) of *D. pentaphyllum* and *A. maritimus* seedlings 8 months after planting (mean ± standard deviation, n = 5).

Parameters	*D. pentaphyllum*	*A. maritimus*
Dehydrogenase (μg INTF g^{-1} soil)		
C	6.3 ± 0.7	11.0 ± 2.4
A	11.6 ± 2.0	18.1 ± 0.2
M	8.3 ± 0.7	12.3 ± 0.4
A × M	16.4 ± 1.6	18.8 ± 2.5
Acid pase (μmol PNP g^{-1} soil h^{-1})		
C	2.3 ± 0.3	1.4 ± 0.1
A	2.3 ± 0.4	1.4 ± 0.1
M	2.5 ± 0.4	1.5 ± 0.1
A × M	2.6 ± 0.3	1.6 ± 0.1
β-Glucosidase (μmol PNP g^{-1} soil h^{-1})		
C	0.3 ± 0.1	0.3 ± 0.1
A	0.6 ± 0.1	0.6 ± 0.1
M	0.6 ± 0.1	0.5 ± 0.1
A × M	0.6 ± 0.1	0.6 ± 0.1
Available P (μg g^{-1} soil)		
C	11 ± 2	15 ± 1
A	15 ± 2	18 ± 2
M	13 ± 2	15 ± 2
A × M	17 ± 4	21 ± 2
Total N (g kg^{-1} soil)		
C	0.57 ± 0.10	0.66 ± 0.11
A	1.23 ± 0.12	1.09 ± 0.19
M	0.80 ± 0.15	0.60 ± 0.12
A × M	1.23 ± 0.20	1.15 ± 0.17

Pase: phosphomonoesterase.

Table 5
Heavy metal concentrations in shoot in response to addition of fermented sugar beet residue (A) and AMF inoculation (M) of *D. pentaphyllum* and *A. maritimus* seedlings 8 months after planting (mean ± standard deviation, n = 5).

Parameters	*D. pentaphyllum*	*A. maritimus*
Cd (μg g^{-1})		
C	0.2 ± 0.1	0.8 ± 0.1
A	0.2 ± 0.0	1.0 ± 0.1
M	0.2 ± 0.0	0.8 ± 0.2
A × M	0.3 ± 0.1	0.8 ± 0.3
Cu (μg g^{-1})		
C	5.3 ± 2.1	10.2 ± 1.3
A	6.0 ± 0.6	12.0 ± 1.2
M	5.0 ± 0.8	10.9 ± 2.6
A × M	10.6 ± 2.3	11.8 ± 2.0
Pb (μg g^{-1})		
C	18 ± 11	64 ± 20
A	9.6 ± 3	177 ± 71
M	29 ± 17	91 ± 31
A × M	19 ± 5	81 ± 54
Zn (μg g^{-1})		
C	38 ± 12	140 ± 33
A	40 ± 4	228 ± 46
M	46 ± 17	157 ± 43
A × M	56 ± 7	162 ± 43

3.4. Effects of the amendment and AM fungus on the rhizospheric microbial community parameters

Excepting the AMF-related NLFA, the addition of the SBR promoted all microbial groups studied in the rhizosphere of *D. pentaphyllum*, increasing the total PLFA ($P = 0.003$), saprophytic fungi-related PLFA ($P = 0.019$), Gram+ related PLFA ($P < 0.001$) and Gram− related PLFA ($P < 0.001$), especially the Gram+ related ones (Tables 3 and 6). In particular, the actinobacterial PLFAs were 4.0-fold more abundant in the amended soil than in the control soil. Coinciding with the mycorrhizal colonisation data, the abundance of the AMF-related NLFA was only increased by the inoculation with *F. mosseae*; this treatment also enhanced the abundance of Gram-related PLFAs ($P < 0.001$).

Table 6
Abundance of signature phospholipids fatty acids and total fatty acid contents in the rhizosphere soil of *D. pentaphyllum* and *A. maritimus* seedlings in response to addition of fermented sugar beet residue (A) and AMF inoculation (M) 8 months after planting (mean ± standard deviation, n = 5).

Parameters	*D. pentaphyllum*	*A. maritimus*
Gram+ PLFA (nmol g^{-1})		
C	2.24 ± 0.98	2.70 ± 0.52
A	6.60 ± 2.51	6.48 ± 0.58
M	3.05 ± 0.30	2.12 ± 0.11
A × M	6.45 ± 0.79	6.78 ± 1.62
Gram− PLFA (nmol g^{-1})		
C	2.69 ± 0.39	1.81 ± 0.31
A	4.51 ± 0.97	3.28 ± 0.85
M	4.26 ± 1.07	1.63 ± 0.47
A × M	5.65 ± 0.86	2.90 ± 0.73
Saprophytic fungi PLFA (nmol g^{-1})		
C	3.06 ± 1.31	2.70 ± 0.64
A	4.76 ± 2.21	4.01 ± 0.81
M	3.90 ± 1.51	2.52 ± 0.26
A × M	4.56 ± 0.77	4.44 ± 1.11
AB PLFA (nmol g^{-1})		
C	0.38 ± 0.01	0.68 ± 0.03
A	1.54 ± 0.48	1.69 ± 0.37
M	0.38 ± 0.05	0.50 ± 0.01
A × M	1.19 ± 0.61	1.24 ± 0.16
AMF NFLA (nmol g^{-1})		
C	4.98 ± 2.11	0.08 ± 0.04
A	4.91 ± 3.98	0.04 ± 0.01
M	19.61 ± 7.16	0.39 ± 0.01
A × M	22.26 ± 12.55	0.42 ± 0.03
Total PLFA (nmol g^{-1})		
C	17.7 ± 7.3	14.2 ± 2.9
A	35.7 ± 9.3	20.1 ± 2.7
M	24.9 ± 8.1	12.3 ± 2.8
A × M	30.8 ± 7.1	24.4 ± 5.6

AB: actinobacteria; AMF: arbuscular mycorrhizal fungi.

Principal component analysis (PCA) of the individual PLFAs measured in the rhizosphere of *D. pentaphyllum* showed that the first principal component (PC1) explained about 42% of the variance while the second one (PC2) explained 26% (Fig. 2). The ANOVA of the two principal components evidenced that the control, amended and inoculated soils differed in their lipid composition ($P < 0.05$, Table 7). The highest values of the first principal component were found for the soil amended with the SBR, alone or in combination with *F. mosseae* (Fig. 2a). This component was characterised by relatively-high loadings of the iso/anteiso methyl-branched fatty acids (i15:0, a15:0, i16:0) markers for Gram+ bacteria, the monounsaturated fatty acids (16:1 ω5, 16:1 ω7) assigned to general bacteria and the cyclic fatty acid (cy17:0) indicator for Gram− bacteria, compared to values of the control soil (Fig. 2b). The second principal component differentiated soils inoculated with the native AM fungus, alone or in combination with SBR from soils amended. According to the loading values, the PLFAs 14:0, 15:0, 17:0 and 17:1 ω8 markers for bacteria and 17:0 10-meth indicator for actinobacteria were responsible for separation along this component (Fig. 2b).

The total amount of PLFA in the rhizosphere of *A. maritimus* plants was only enhanced by the addition of SBR (by 41% compared to control soil, Table 6). The main effect of the amendment was significant for the main microbial groups, enhancing the abundances of saprophytic fungi-related PLFAs ($P = 0.002$) and Gram+ and Gram− bacteria-related PLFAs (Table 3). Likewise, the occurrence of the PLFAs 17:0 10-meth and 18:0 10-meth, considered as specific markers for actinobacteria, increased as a consequence of the addition of SBR. In contrast, these latter markers diminished in

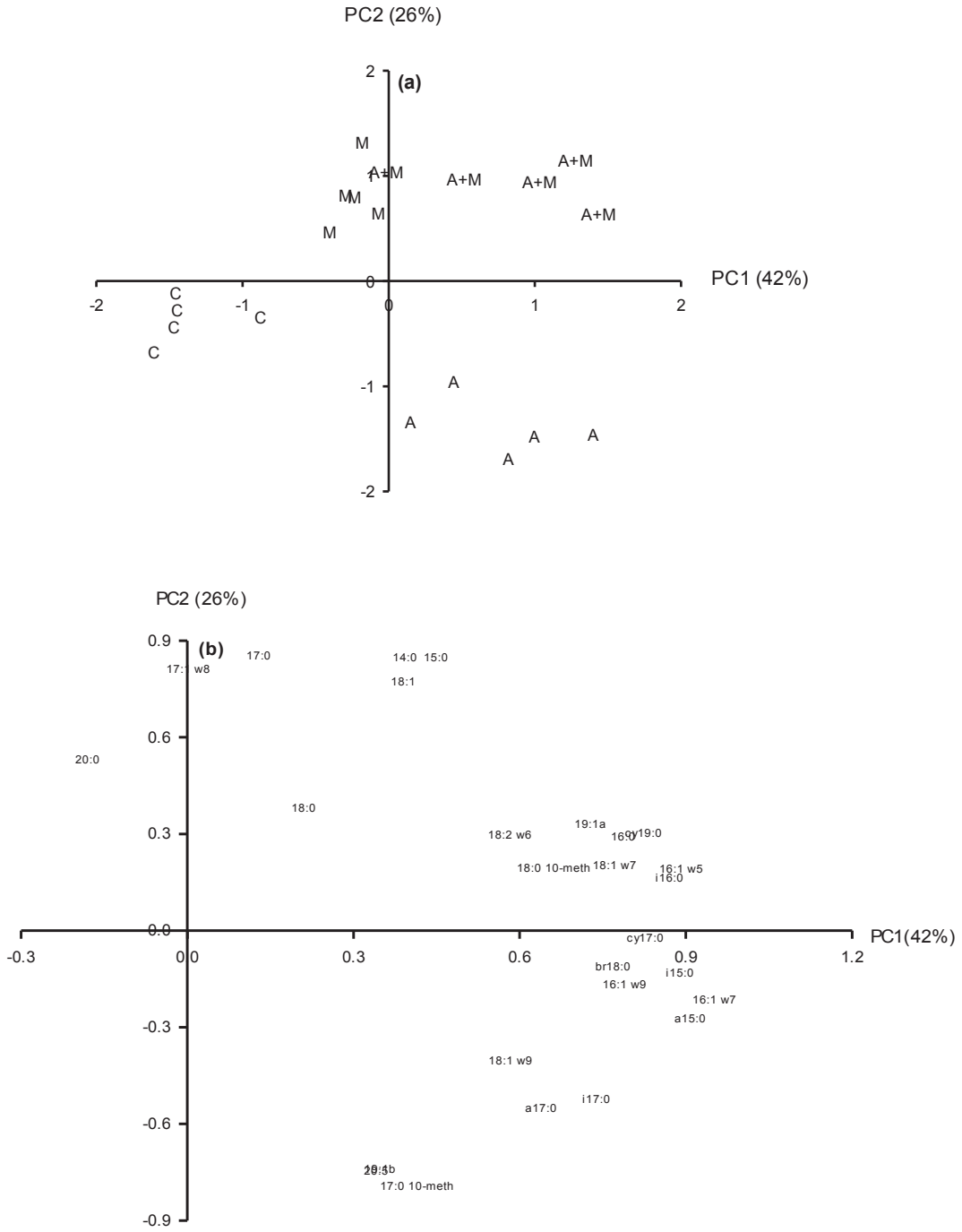

Fig. 2. (a) Score plot from the principal components analysis of PLFA data from soil planted with *D. pentaphyllum*. C, control soil; A, soil with addition of fermented sugar beet residue; M, soil with AM fungus inoculation and A + M, soil with the combined treatment of amendment and AM fungus. (b) Loading values of the first two principal components for individual PLFAs of soil planted with *D. pentaphyllum*.

the soil inoculated with the native AM fungus. The NLFA 16:1 ω5 was significantly ($P < 0.001$) increased by the mycorrhizal inoculation (Tables 3 and 6).

In the PCA of the PLFAs pattern of *A. maritimus*, the first component accounted for 57% of the variance and the second component accounted for 15% (Fig. 3). The ANOVA of the two principal components separated the treatments into two distinct clusters, one comprising the control and inoculated soils and the other the soils amended with SBR, alone or in combination with the native AM fungus ($P < 0.05$, Table 7). The iso/anteiso methyl-branched fatty acids (i15:0, a15:0, i16:0, i17:0, a17:0) markers for Gram+ bacteria, the monounsaturated fatty acid (18:1 ω7) assigned to general bacteria, the cyclic fatty acid (cy17:0) indicator for Gram− bacteria and 10-methyl-substituted fatty acid (18:0 10-meth) assigned to actinobacteria were responsible for separation along the first principal component (Fig. 3b).

In *D. pentaphyllum* and *A. maritimus* plants, the first canonical component (PC1) of the PLFA data was positively correlated with the activities of dehydrogenase ($r = 0.796^{***}$ and $r = 0.869^{***}$, respectively) and β-glucosidase ($r = 0.575^{**}$ and $r = 0.591^{**}$,

Table 7
ANOVA of the loading values of the individual PLFAs for PC1 and PC2 in *D. pentaphyllum* and *A. maritimus* plants.

Treatment	*D. pentaphyllum*		*A. maritimus*	
	PC1	PC2	PC1	PC2
C	a	b	a	c
A	c	a	b	a
M	b	c	a	a
A × M	c	c	b	b

For each component and each plant species, treatments in columns sharing the same letter are not significantly different according to the Tukey's HSD-test ($P < 0.05$).
A, amendment; M, mycorrhiza.

respectively), which confirms a close relationship between the soil microbial community composition and function during the phytomanagement process – as pointed out by Zhang et al. (2006) and Pérez-de-Mora et al. (2006). Likewise, soil nutrient concentrations (available P and total N) were important in determining the composition of the microbial community in the rhizosphere soil of both plants, showing significant positive correlations with the first principal component (for *D. pentaphyllum*: $r = 0.558^{**}$ and $r = 0.921^{***}$, respectively; for *A. maritimus*: $r = 0.589^{**}$ and $r = 0.797^{***}$, respectively). However, the PLFAs patterns were not affected by the presence of soluble heavy metals in the soil.

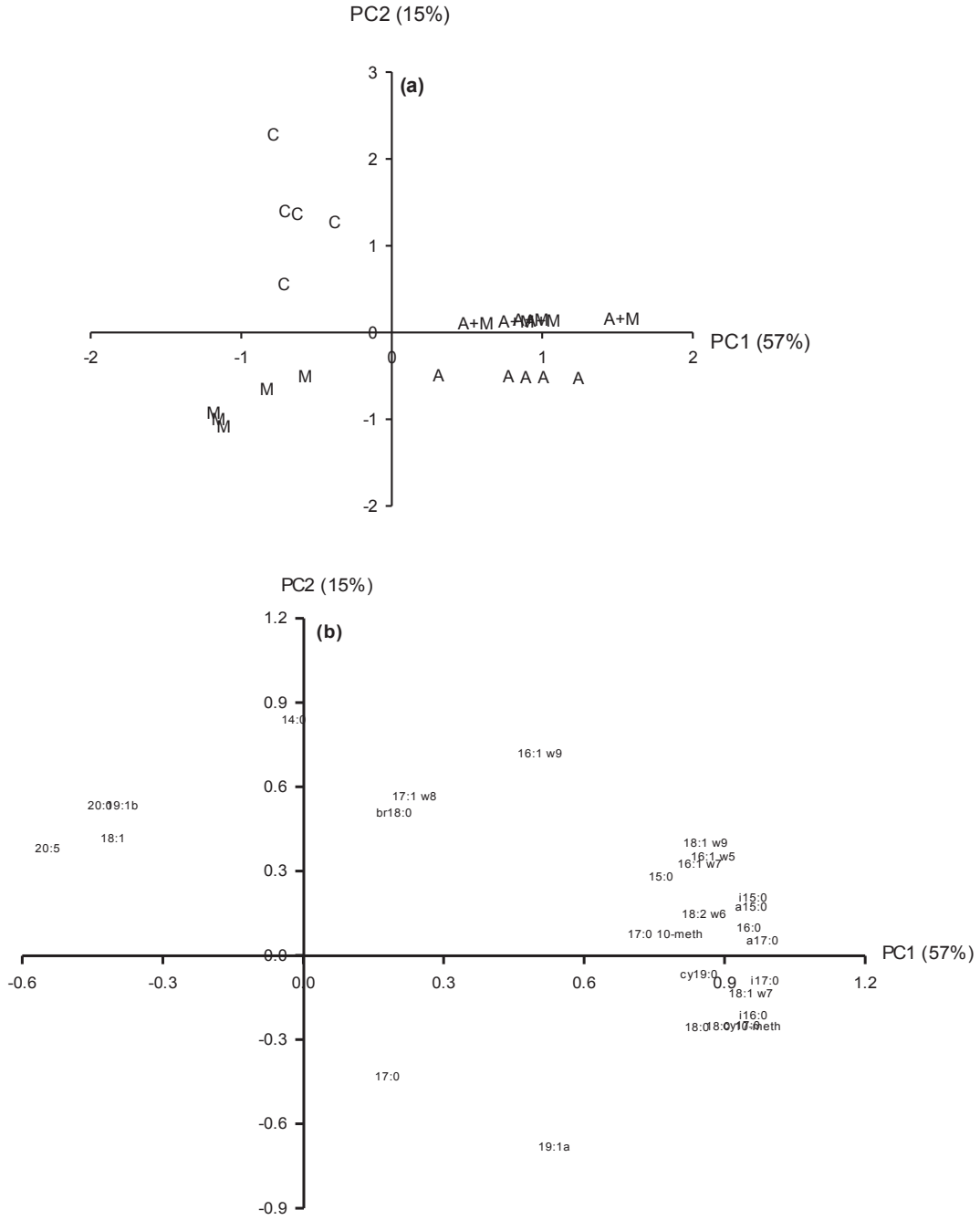

Fig. 3. (a) Score plot from the principal components analysis of PLFA data from soil planted with *A. maritimus*. C, control soil; A, soil with addition of fermented sugar beet residue; M, soil with AM fungus inoculation and A + M, soil with the combined treatment of amendment and AM fungus. (b) Loading values of the first two principal components for individual PLFAs of soil planted with *A. maritimus*.

4. Discussion

4.1. Effect of the treatments on plant growth

The combined treatment, involving AM fungal inoculation and organic amendment, did not produce an additive or synergistic effect on the growth of *D. pentaphyllum* or *A. maritimus* plants under the local semiarid conditions. In contrast, the combination of the two treatments increased the growth of *D. pentaphyllum* to a lesser extent than each treatment applied individually. In our study, the growth disadvantage conferred on the host plant by the combined treatment cannot be due to an adverse effect of the amendment on the formation and activity of the AMF because it has been shown that the toxic substances contained in the SBR – like ferulic acid – are eliminated during the fermentation process (Medina et al., 2011). These results contrast with those obtained by other authors (Medina and Azcón, 2010; Wang et al., 2013), who found a synergistic effect of organic amendment and AMF with respect to the improvement of plant growth in pot experiments. In addition, we have recently found that the combination of urban organic waste compost and a native mycorrhizal fungus was effective at promoting the establishment of the legume shrub *Anthyllis cytisoides* under field conditions (Kohler et al., 2015). Although the mycorrhizal colonisation of *D. pentaphyllum* roots did not change following the combined treatment, it has been shown that the AMF diversity of a metal-contaminated soil may be altered after organic residue addition (Alguacil et al., 2011). In consequence, the resulting fungal community might provide less benefit to its host plants in the amended soil. Thus, the effectiveness of combining amendment addition and mycorrhizal inoculation to promote the growth of host plants depends upon the specific plant as well as on the type of organic residue assayed.

This study confirms the ability of SBR to stimulate plant growth and nutrition in a semiarid contaminated soil, coinciding with the results obtained previously using this organic amendment under controlled conditions (Carrasco et al., 2009; Fernández et al., 2012). However, *D. pentaphyllum* and *A. maritimus* showed different levels of response to the addition of the amendment. The fermented SBR was more effective at increasing shoot biomass production for *D. pentaphyllum* than for *A. maritimus*. This might be related to the different morphological and physiological strategies developed by the two plant species to cope with the long and severe drought characteristic of the experimental area. The reduction of leaf area and stem dry mass in response to induced water stress has been described in *A. maritimus* plants as an avoidance mechanism (Rodríguez et al., 2005). The increased root/shoot ratio of *A. maritimus* plants grown in the amended soil, compared to control plants, could be considered as another mechanism of drought tolerance (Lansac et al., 1995). However, the addition of SBR increased the shoot and root biomass of *D. pentaphyllum* in proportion, so that there was no change in the root/shoot ratio. The benefits of organic amendments with regard to improving the plant growth in contaminated soils may be related also to their ability to render heavy metals less bioavailable in soil, through complexation with the reactive fraction of organic substrates; as a result, their uptake from the soil by plants is reduced (van Herwijnen et al., 2007). The Cd, Cu, Pb and Zn concentrations in shoots of *D. pentaphyllum* grown in the amended soil were within the normal ranges for plants (Kabata-Pendias and Pendias, 2001). However, the SBR provoked excessive or potentially-toxic levels of Zn in the above-ground parts of the halophyte *A. maritimus*, which could constitute a risk through their entry into the food chain (Kabata-Pendias and Pendias, 2001).

The effect of the inoculation with a native AM fungus on plant growth depended on the plant species, being only effective in the establishment of *D. pentaphyllum* plants. This same *F. mosseae* strain was also effective at stimulating the growth of the leguminous shrub *Coronilla juncea* in the same contaminated soil (Carrasco et al., 2011). It has been demonstrated that different plant species show different degrees of susceptibility to colonisation by AMF in soils affected by metal contamination (Alguacil et al., 2011). In the present study, colonisation by the local AMF community differed significantly between the two species. Thus, the mycorrhizal colonisation in roots of *D. pentaphyllum* was 6.4-fold higher than in those of *A. maritimus*. This shows that *D. pentaphyllum* is highly mycotrophic in the local semiarid conditions, which is also reflected in the approximately 39% higher acid phosphomonoesterase activity in the rhizosphere of *D. pentaphyllum*. We have previously demonstrated that mycorrhizal colonisation is important for nitrate assimilation activity and for mitigating the detrimental effects of drought in *D. pentaphyllum* plants grown in dry soils (Caravaca et al., 2003).

4.2. Effect of the treatments on the soil microbial community composition and function

The recovery of composition and functions of soil microbial community may depend on the type of vegetation utilised for phytostabilisation, as the quantity and quality of litter and the production of root exudates affect the proliferation of different microbial communities in the surrounding soil (Jassey et al., 2013). Interestingly, the effects of the mycorrhizal inoculum on the microbial community composition depended on the type of plant. Thus, the PCA of the PLFA patterns demonstrated that the native AM fungus caused shifts in the microbial community composition of the rhizosphere of *D. pentaphyllum*, whilst it had no effect on that of the rhizosphere of *A. maritimus*. In particular, the abundance of specific FA biomarkers for Gram− bacteria was higher in the rhizosphere of plants inoculated with *F. mosseae* than in the control soil, although without differences with respect to the amended soil. The lack of effect of the mycorrhizal inoculation in the rhizosphere of *A. maritimus* could be related to the low degree of mycorrhizal colonisation in its roots.

For both plant species the SBR-amended soils showed different PLFAs patterns relative to the control soil, which points to different associated microbial communities. The soil microbial community composition may be altered as a result of extra nutrients provided by the amendment in addition to its intrinsic microbial community (Farrell et al., 2010). The community composition of bacteria shifted in the amended soils, in particular that of the Gram+ bacteria. A similar trend has been recorded previously, when using organic amendments for the remediation of heavy-metal-polluted soils (Farrell et al., 2010; Fernández et al., 2012). However, a predominance of Gram− over Gram+ bacteria is often found in heavy-metal-contaminated soils due to their better adaptation to adverse conditions (Carrasco et al., 2010). Then, the increased abundance of indicators of Gram+ bacteria along the neutral character of the mine tailing could reveal that the toxic effect of the heavy metals on the microbial populations was reduced or eliminated after the addition of the amendment. The major presence of Gram+ bacteria in the amended soil could have a positive effect on the proliferation of AMF (Artursson et al., 2006). In our study, the fermented SBR did not affect the abundance of the NLFA 16:1ω5 used as a biomarker for AMF biomass, although this same amendment changed the composition of AM fungal communities in a heavy-metal-polluted soil in a semiarid area (Alguacil et al., 2011). This agrees with the results obtained by Börjesson et al. (2014) after the addition of sewage sludge to a heavy-metal-polluted soil under field conditions in a temperate climate.

In conclusion, in the present study the effectiveness of the

inoculation with a native AM fungus and the addition of fermented SBR as phytomanagement technologies for a contaminated soil under semiarid conditions, depended on the plant species. The amendment was effective at promoting growth of *D. pentaphyllum* and *A. maritimus*, whilst the mycorrhizal inoculation was only suitable for the establishment of the leguminous shrub. The PLFA results indicate that the amendment induced shifts in the microbial community composition, with increases in key components of the biomass such as bacteria, actinobacteria and saprophytic fungi, resulting in apparently fully-functional microbiota probably contributing to nutrient turnover and plant development. Increased soil nutrient concentrations and stimulated soil microbial function were significant in determining the soil microbial community composition of the amended soil. Shifts in the microbial community composition promoted by the AM fungus were related to the enhanced abundance of specific FA biomarkers for Gram− bacteria and AMF in the inoculated plants. The composition and function of the microbial community could be used as useful indicators to assess the improvement of the microbial component of ecosystems following the application of phytomanagement technologies.

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